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(54) Title: KERATOCONJUNCTIVITIS CYTOTOXIN AND METHODS FOR ITS USE AND PRODUCTION

(57) Abstract

This invention relates to a cytotoxin from *Moraxella bovis* substantially free of intact microbial cells, which is toxic to bovine peripheral blood neutrophils and which lacks hemolytic activity. This cytotoxin is useful as a vaccine for protection of animals from infectious bovine keratoconjunctivitis. The cytotoxin is further characterized in that it is not capable of hydrolyzing casein, it is net negatively charged at a pH of 7.4 and by its ability to elute off of a DEAE exchange column with a 3ml gel bed under a salt gradient at a 0.2M to about a 0.3M salt concentration. Further, the cytotoxic activity to bovine peripheral blood neutrophils is sensitive to zinc salts. Typically, the cytotoxin is prepared by clarifying and purifying culture filtrates of *Moraxella bovis*.

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KERATOCONJUNCTIVITIS CYTOTOXIN AND METHODS FOR ITS USE AND PRODUCTION

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a keratoconjunctivitis cytotoxin, to methods for extracting and purifying the cytotoxin and to veterinary vaccines comprising the cytotoxin.

Infectious bovine keratoconjunctivitis (IBK) is a serious disease of the eyes of cattle commonly known as pinkeye. It is caused by the bacteria Moraxella bovis. Moraxella sp. are aerobic, gram-negative rods appearing in pairs of short chains. They belong to the Neisseriaceae family and are oxidase (+) and catalase (-).

IBK is highly contagious and can be transmitted rapidly throughout a herd. Acute to chronic inflammation of the eye occurs which impairs the animal's sight. This disease causes great financial loss in the cattle industry because it is a debilitating disease which affects cattle of all ages and breeds.

Although IBK was first described a century ago, the pathogenesis of the disease is not yet understood. Moraxella bovis is considered to be the principal infectious agent associated with IBK. The earliest described ocular changes after inoculation of M. bovis into the eyes of cattle include necrosis and sloughing of the corneal epithelium, and bacteria lying in pit-like defects on the surface of the epithelial cells. The margins of these pit-like defects conform closely to the shape of the associated bacterial cell, indicating that epithelial cell cytotoxicity may constitute an important pathogenic mechanism in the early stages of IBK.

Macroscopically visible corneal ulcers may occur by 4 to 8 days after infection. At that time, the corneal stroma underlying the ulcerated area is infiltrated by bacteria and neutrophils. Many of the neutrophils and epithelial cells in the ulcerated areas appear necrotic. The contribution of these inflammatory cells to the corneal lesions of IBK is unclear; however, studies have indicated that degenerating polymorphonuclear leukocytes release enzymes into the cornea which may increase the size of ulcers and retard healing. Thus, a toxin effect of M. bovis upon neutrophils could result in the release of endogenous mediators capable of increasing corneal destruction. Cytotoxicity by M. bovis also would confer antiphagocytic effects upon bacterium. Although previous studies have shown that M. bovis is toxic to bovine monocytes in vitro, the cytotoxic effect of the bacterium on neutrophils was previously unknown.

Treatment of an animal infected with IBK is difficult and impractical as current treatment methods are not generally cost effective. Further, treatment is complicated if the disease occurs in a range herd. Thus, preventative treatment through the use of a vaccine would be highly desirable. The toxin disclosed herein is useful for a preventative vaccine.

SUMMARY OF THE INVENTION

This invention relates to a cytotoxin substantially free of intact microbial cells from Moraxella bovis which is toxic to bovine peripheral blood neutrophils and which lacks hemolytic activity. This cytotoxin is useful as a vaccine for protection of animals from infectious bovine keratoconjunctivitis. The cytotoxin is further characterized in that it is not capable of hydrolyzing casein, it is net negatively charged at a pH of

7.4 and by its ability to elute off of a DEAE exchange column with a 3ml gel bed under a salt gradient at a 0.2M to about a 0.3M salt concentration. Further, the cytotoxic activity to bovine peripheral blood neutrophils is sensitive to zinc salt. Typically, the cytotoxin is prepared by clarifying and purifying culture filtrates of Moraxella bovis.

DETAILED DESCRIPTION

This invention relates to a cytotoxin derived from M. bovis which is cytotoxic to bovine peripheral blood neutrophils and which can be used as a vaccine against keratoconjunctivitis. Methods for purifying the cytotoxin are also disclosed.

Extraction and Purification of the Cytotoxin

Moraxella bovis strains useful in preparing the cytotoxin of the invention can be isolated from clinical cases of IBK or can be obtained from public sources. Public sources include, for example, the American Type Culture Collection in Rockville, Maryland, U.S.A. (ATCC) where M. bovis strains are deposited under accession numbers 10900, 25576, 17947 and 17948. The most preferred strain is Tifton 1 strain, deposited with the ATCC on January 10, 1989 having accession number 53854. The bacteria will grow on most common bacterial culture media.

M. bovis grown on McCoy's media (Grand Island Biologicals, Grand Island, New York or Flow Labs, Inglewood, California) supplemented with about 3% bovine serum albumin (BSA) and about 3% fetal calf serum yields filtrates with high levels of cytotoxic activity. However, for cytotoxin purification purposes, it is preferred that a minimal medium be used which contains fewer exogenous proteins. Dialyzed GC media is a preferred minimal medium which yields good quantities of purified cytotoxin. Dialyzed GC media

contains all components of GC media which is commercially available except that high molecular weight proteose peptone is substituted with proteose peptone which has been solubilized in water and dialyzed to remove high MW peptones, typically those greater than about 14,000MW. One liter of dialyzed GC media contains dialysate from 15g of powdered proteose peptone.

Cytotoxin production is best if the bacteria is grown on agar plates rather than in liquid shake cultures. Agar plates or shake cultures are preferably incubated at a temperature of 37°C for about 18 to about 36 hours.

After incubation, the bacteria is harvested from the plates by washing the agar surface with an inorganic buffer having a molarity of about 0.01 to 0.05 and a pH of 7.4. The preferred buffer is Hepes and 200mM $CaCl_2$. Preferably, the buffer is added to the agar plates in a ratio of about 5ml:75cm^2 to about 15ml:75cm^2 of agar surface area.

The culture filtrates are clarified by removing the solid materials by differential centrifugation, ultrafiltration or the like. Typically, the materials are centrifuged and the supernatant is then further purified by passing through a polycarbonate filter, preferably of about 0.22μ size.

The crude filtrate is next precipitated by using standard techniques known in the field, such as the use of cold acetone or salts. Concentrations and other conditions vary with the salt chosen. Standard experimental strategies can be used to optimize selective precipitation for each salt. Selective precipitation using solutes is preferred. The solute most preferred is 70% ammonium sulfate. The filtrate may be centrifuged to collect the precipitate. The precipitate is then collected and redissolved in a suitable buffer as described above. After the precipitation of the

cytotoxin and resuspension in buffer solution, it has been found to be most advantageous to pass the preparation through a molecular filtration device such as a Bio Gel PD-10 column (Bio-Rad Laboratories, Richmond, California). The first protein peak is collected and diluted at about 1:2 to about 1:10, most preferably about 1:10, in a buffer solution, as described above.

The preparation is then further purified using standard column chromatographic techniques which may include, for example, ion exchange chromatography, molecular filtration, electrophoresis, or isoelectric focusing. Standard columns generally recognized for use in purifying proteins are contemplated in purifying the cytotoxin of the present invention and many alternative columns are commercially available and known to those skilled in the art.

The filtrate buffer solution is then placed on an anion exchange column such as those produced by Sigma Chemical Co., St. Louis, Missouri. The anion exchange column is preferably a DEAE-Sephadex, DEAE-Sephacryl or DEAE cellulose column having about a 1ml to 10ml gel bed. A DEAE column with about a 3ml gel bed is most preferred. The cytotoxin is eluted from the column through the use of a salt gradient. Preferably, the cytotoxin solution is bound to the column, the column is then washed with buffer and then a salt gradient is passed through the column. The salt gradient may be of any range such that the range encompasses at least a 0.0M to 0.3M solution of a salt such as NaCl. The purified cytotoxin elutes off of a DEAE exchange column under a salt gradient at about 0.2M to about 0.3M NaCl concentration.

Using the method described below, eluate fractions may be individually tested to determine which fractions are

cytotoxic to bovine peripheral blood neutrophils and which lack hemolytic activity.

Characterization of the Cytotoxin

The M. bovis cytotoxin of this invention is substantially free of intact microbial cells which means that it has been derived from M. bovis cultures and filtered or otherwise purified so that it is about preferably 95% to 100%, most preferably 100%, free of intact microbial cells.

The M. bovis cytotoxin is cytotoxic to bovine peripheral blood neutrophils. This toxicity is measured by observing the release of chromium by the peripheral blood neutrophils upon subjection of the neutrophils to the cytotoxin pursuant to the methodology described by Miggiano et al., Transpl. Proc., 4:231-237 (1972); Kurtz et al., Transfusion, 19:398-403 (1979); and Carlson et al., Proc. Soc. Exp. Biol. Med., 142:853-856 (1973). The preferred method is set forth in Example II below.

The M. bovis cytotoxin is further characterized by its lack of hemolytic activity. Hemolytic activity can be assayed by methods known in the art such as that described by Bradley et al., J. Investig. Derm., 78:206-209 (1982). The preferred method is that given in Example II below.

The cytotoxin is not capable of hydrolyzing casein. The phrase "not capable of hydrolyzing casein" means that the cytotoxin, when placed in contact with a milk substrate, does not hydrolyze the casein and create clear zones in the media. Casein activity assays are described and well-known in the art as, for example, in U.S. Patent No. 4,675,176, which is incorporated by reference herein.

The cytotoxin is further characterized in that its cytotoxicity to bovine peripheral blood neutrophils is inhibited by zinc salts, such as zinc sulfate. When the cytotoxin is placed into contact with a zinc salt at a final

concentration of 2mM its cytotoxicity to the neutrophils is inhibited.

The cytotoxin has a molecular weight of about 42KD based on polyacrylamide gel electrophoresis (PAGE). The cytotoxicity of the cytotoxin to bovine peripheral blood neutrophils is not inhibited when mixed with PMSF (40μg/1ml phenylmethane sulfonyl fluoride), pepstatin (0.7μg/ml), TLCK (50μg/ml, 1-1-chloro-3- [4-tosyl amido] -7-amino-2-heptanone-HCl), phosphoramidon (50μg/ml) or leupeptin (0.5μg/ml) either singly or together as a cocktail, all obtained from Boehringer Mannheim, Indianapolis, Indiana.

The toxin is heat labile, being rapidly inactivated by heating at 56°C for over 15 minutes. It is a net negatively charged molecule at a pH of 7.4.

The cytotoxins described herein from Moraxella bovis further include those which are immunologically equivalent to the cytotoxins derived from M. bovis culture filtrates. By immunologically equivalent, it is meant that the cytotoxin embraces those that are immunologically indistinct to the immune systems of cattle. Such cytotoxins would include proteins identical to the cytotoxins described herein as well as those comprising minor modifications to the primary sequence of the protein, such as amino acid deletions, additions, substitutions or chemical modifications thereto (e.g., alkylation, reduction). The cytotoxins can be obtained from the cell cultures of M. bovis, but can also be derived from chemical synthetic means or recombinant genetic technology. To determine immunological equivalence, one can use any number of standard immunological assays. The most convenient assay would involve competitive immunoassays wherein equivalence could be determined when antibodies against naturally secreted cytotoxin bind in about the same manner to about

the same extent as the cytotoxin being assayed for equivalence.

Vaccines Using the Cytotoxin

A vaccine prepared utilizing the purified cytotoxin or its immunogenic equivalents thereof can be prepared in vaccine dose form by well-known procedures. The vaccine can be administered intramuscularly or subcutaneously. For parenteral administration, such as subcutaneous injection, the immunogen may be combined with a suitable carrier, for example, it may be administered in water, saline or buffered vehicles with or without various adjuvants or immunomodulating agents such as aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, Corynebacterium parvum (Propionobacterium acnes), Bordetella pertussis, polyribonucleotides, sodium alginate, lanolin, lysolecithin, vitamin A, saponin, liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.). Other suitable adjuvants are Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Michigan).

The proportion of immunogen and adjuvant can be varied over a broad range so long as both are present in effective amounts. For example, aluminum hydroxide can be present in an amount of about 0.5% of the vaccine mixture (Al_2O_3 basis). On a per-dose basis, the concentration of the cytotoxin can range from about 1μg to about 2,000μg per bovine host. A preferable range is from about 100μg to about 1,000μg per dose. A suitable dose size is about 1-10ml, preferably about 1.0ml. Accordingly, a dose for

intramuscular injection, for example, would comprise 1ml containing 1.0mg of immunogen in admixture with 0.5% aluminum hydroxide. Comparable dose forms can also be prepared for parenteral administration to calves, but the amount of immunogen per dose will be smaller, for example, about 1μg to about 500μg per dose.

For the initial vaccination of immunologically naive cows, a regimen of between 1 and 4 doses can be used with the injections spaced out over a two- to six-week period. Typically, a two-dose regimen is used. The second dose of the vaccine then should be administered some weeks after the first dose, for example, about two to four weeks later. Animals that have been previously exposed to M. bovis or have received colostral antibodies from the mother may require booster injections. A two-dose regimen is considered preferable for the most effective immunization of the calves. Semiannual revaccination is recommended for breeding animals. Calves may be vaccinated at about 1-3 months after birth, again at four to six months, and yearly or preferably semi-annually thereafter.

The vaccine may also be combined with other vaccines for other diseases to produce multivalent vaccines. It may also be combined with other medicaments, for example, antibiotics. A pharmaceutically effective amount of the vaccine can be employed with a pharmaceutically acceptable carrier or diluent understood to be useful for the vaccination of animals such as swine, cattle, sheep, goats, and other mammals.

Other vaccines may be prepared according to methods well-known to those skilled in the art as set forth, for example, in I. Tizard, "An Introduction to Veterinary Immunology," 2nd Ed (1982), which is incorporated herein by reference.

The vaccine or the cytotoxin may be stored in lyophilized form. Though cytotoxic activity may be lost, the antigenic properties of the cytotoxin are maintained.

EXAMPLES

I. CULTURING M. BOVIS AND CYTOTOXIN PRODUCTION

A. Bacterial Isolates

The hemolytic isolate of M. bovis for these experiments was obtained from a cow with IBK in Georgia (Tifton 1). The bacterial isolate was maintained as lyophilized stock cultures. This isolate was positively identified as M. bovis using biochemical and morphologic criteria described in Smith and George, Am. J. Vet. Res., 46:804-807 (1985) which is incorporated by reference herein.

B. Preparation of dialyzed GC Media

1. 15g of proteose peptone #3 were solubilized in 50ml of distilled water 24 hours prior to making the media.

2. Six strips of dialysis tubing (6MW to 14,000MW), 40cm to 45cm long were cut and moistened in distilled water. Each was filled with 12ml of the solubilized proteose peptone.

3. The sacs were sealed at both ends and washed thoroughly in distilled water. The sacs were then placed in a graduated cylinder with parafilm to seal the top. The cylinders were then placed in the refrigerator. Dialysis was allowed to occur for 24-36 hours in the cold.

4. After dialysis, the sacs were removed. Distilled water was added to the dialysate to give a final volume of 1 liter. Sodium chloride NaCl (5g) corn starch (1g), potassium phosphate (dibasic, 4g), potassium phosphate (monobasic, 1g), and agar (10g) were added in a 2-liter erlenmeyer flask. The flask was placed on a hot plate with constant stirring set at a heat setting of 6 until the agar

was melted -- about 15-20 minutes. The flask was equipped with a cotton, gauze plug and aluminum foil stopper and was autoclaved for 18 minutes, no dry, liquid cycle. After autoclaving the media was put in a 56°C waterbath until the temperature was equilibrated.

5. 5ml of Hepes buffer (0.02M Hepes, 100mg/l of calcium chloride) was pipetted into each of five, 15-ml screwcap pyrex tubes. Caps were screwed on each tube.

6. Isovitalex™ bottles were obtained from Becton-Dickinson Microbiology Systems. These bottles contain ingredients important for growing Gonorrhea coccus, thus the reason for the name GC agar. Typically, these bottles contain the following ingredients: vitamin B_{12}, L-glutamine, guanine HCl, PABA, cocarboxylase adenine, diphosphoparadine nucleotide, oxidized (coenzyme 1), ferric nitrate, thiamine, L-cysteine HCl, and L-cystine. The Isovitalex™ liquid was combined with the lyophilized powder per the instructions. After the powder was dissolved, the fluid was aspirated.

7. The agar, cooled to 56°C, was removed from the waterbath. The entire contents of the Isovitalex™ bottle was added to the erlenmeyer flask. About 300ml of agar were poured into each petri plate. The agar was allowed to harden before inoculation.

8. 2-10 "fried egg" type of M bovis colonies were inoculated into the sterile Hepes solution. A 5cc pipette was used to swish the fluid back and forth one or two times. 2-5ml of the inoculated buffer was pipetted onto each agar surface. The inoculum was spread onto this agar surface using a sterile glass hockey stick. The plates were wrapped and incubated for 24 hours at 37°C. The plates were

ready for harvest and were all checked for purity of growth.

C. Purification of the Cytotoxin

1. The agar surface of each plate was flushed with 10ml of Hepes $CaCl_2$ (0.02M, 100mg/l, pH 7.4) per $75cm^2$ of the agar surface area. A sterile glass hockey stick was used to remove the bacterial growth. The harvested culture was pipetted into a 50-ml centrifuge tube. The tubes were centrifuged for 15 minutes at 3,000rpm. The supernatant was removed and filter-sterilized two times through a 0.22-micron polycarbonate filter (47mm diameter, Nucelopore, Inc., Pleasanton, California).

2. The sterile filtrate then was diluted 1:2 in the low ionic strength Hepes buffer, as described above, and 40ml was pumped onto a 3ml gel bed DEAE column (DEAE Spectra gel M, Spectrum Medical Industries Inc., Los Angeles, California) (1.0ml/minute). After the toxin filtrate was applied, the column was washed in 30ml of buffer. The proteins were eluted from the column differentially by application of 30ml of Hepes buffer containing 0.1ml of NaCl, followed by a salt gradient (0.1M to 0.3M, 30ml in Hepes buffer).

3. The eluate was removed in 3ml fractions and each fraction was tested for cytotoxic and hemolytic activity as described below. Hemolysin eluted at approximately 0.18M NaCl concentration. Cytotoxin eluted at 0.22M NaCl concentration. Fractions containing the cytotoxin consistently showed a single SDS polyacrylamide gel electrophoresis protein band with an apparent molecular weight of 42KD. The fractions containing the hemolysin contained a large number of proteins.

II. CHARACTERISTICS OF THE CYTOTOXIN

A. Tests for Cytotoxicity and Hemolytic Activity

Cytotoxicity tests were done by challenging bovine peripheral blood neutrophils with solutions containing the cytotoxin as described below:

1. Neutrophil Preparation

Bovine peripheral blood neutrophils were isolated by modification of methods described by Carlson et al., Proc. Soc. Exp. Biol. Med., 142:853-856 (1973) and Chambers et al., Vet. Immunopath., 5:197-202 (1983). Heparinized blood (40ml) was collected from the jugular vein of either of 2 normal adult donors. After collection, 10ml aliquots of blood were centrifuged (4°C, 500xg) for 15 minutes. After centrifugation, the plasma was aspirated and discarded. Then, 2ml of the buffy coat and topmost layers of the packed erythrocytes was removed from each tube, and pooled. The erythrocytes in the pooled sample were lysed osmotically by the addition of 40ml of cold distilled water. The cell suspension was gently agitated for 20 seconds, and then hemolysis was stopped by addition of 20ml of cold hypertonic phosphate buffered saline solution (2.7% NaCl, 0.007M phosphate buffer, pH 6.8). The cell suspension then was centrifuged for 10 minutes (4°C, 175xg). After centrifugation, the pellet was resuspended in 8ml of phosphate buffered saline solution (PBSS, 0.007M, pH 6.8, 0.9% NaCl). The suspended cells then were layered over 4.0ml of lymphocyte separation media (density 1.077 to 1.080g/ml, Litton Bionetics Inc., Kensington, Maryland) and the mixture was centrifuged for 25 minutes (4°C, 500xg). After centrifugation, the supernatant was discarded, and the cell pellet was washed three times in cold PBSS. Just prior to the final wash, the cells were counted using a hemocytometer and the viability was determined by staining with 0.1% eosin dye in phosphate buffered saline. After

washing, the cells were resuspended and adjusted to a final concentration of $2x10^7$ neutrophils/ml by the addition of PBSS. The yield from each 40ml blood sample was approximately $5x10^7$ to $7x10^7$ cells, and was composed of 80% to 90% neutrophils. More than 90% of the cells were viable. Human neutrophils were purified by the Ficoll-Hypaque and dextran density gradient sedimentation method as described by Boyum, Scand. J. Clin. Lab. Invest., 21 Supp.97:77-89 (1968).

2. Chromium Release Studies as an Indication of Cytotoxicity

Isotope labeling of the neutrophils was performed according to Kurtz et al., Transfusion, 19:398-403 (1979). The purified neutrophil suspensions were incubated with ^{51}Cr labeled Na_2CrO_4 (100 $\mu C/10^7$ neutrophils, 37°C, 1 hour, ICN Inc., Irvine, California). During incubation, the cells were rotated (Roto-Torque, Cole Palmer, Chicago, IL). After incubation, the cells were washed three times in cold PBSS and were finally resuspended in McCoy's to a final concentration of 1×10^6 neutrophils/ml before the radioactivity in 3 aliquots (0.5ml) of each cell suspension was counted (Autogamma Scintillation Spectrometer, Hewlett Packard, Palo Alto, California).

The cytotoxicity assays were performed by incubating 0.5ml of the labeled neutrophils at 37°C with 0.5ml of the preparation to be assayed (crude M. bovis filtrates or partially purified fractions) for 30 minutes with gentle rotation. When fractions were taken from the DEAE exchange column as described in Example I.C.3. above, 0.5ml of each 3ml fraction was promptly incubated with the neutrophils after elution. Incubation of the labeled cells in McCoy's served as a negative control. After incubation, the cells were sedimented by centrifugation (200xg, 4°C) for 10 minutes, and the radioactivity in 0.5ml of the

supernatant was measured. Data reflected the mean of duplicate determinations, and were recorded as a percent of ^{51}Cr release, using a modification of a recommended expression (Miggiano et al., Transpl. Proc., 4:231-237 (1972)):

$$\% \text{ Release} = \frac{\text{cpm sample} - \text{cpm media controls} \times 2}{(\text{cpm neutrophils} - \text{cpm media controls})} \times 100$$

Table I below shows the cytotoxic activities of fractions taken off the DEAE column.

3. Hemolysin Activity

Hemolysin assays were performed in phenol-red free McCoy's media (25mM Hepes buffer, Gibco Inc., Grand Island, New York) using the method described by Ostle et al., Am. J. Vet. Res., 45:1848-1851 (1984). Hemolysin release was determined by the following protocol:

a. Filtrate or eluate fractions from Example I.C.3. above to be tested were obtained.

b. A 0.5ml aliquot of the filtrate or eluate fraction was mixed with 0.01 ml of 3x washed packed erythrocytes (RBC) washed 3x in McCoy's media. The suspending buffer was sterile 0.017M PBSS in NaCl (pH7.4) with 2.0 mM $CaCl_2$ making the final RBC concentration 2%. When eluate fractions were taken from the DEAE column, each fraction was promptly incubated with the erythrocytes.

c. Controls were prepared which consisted of the washed RBC's and 0.5ml of sterile McCoy's medium.

d. All samples were incubated at 37°C for 8 hours.

e. The samples were spun immediately, and the supernatant was tested for peroxidase activity.

f. Peroxidase activity was tested by adding 3.0ml of reaction mixture to 0.20ml of supernatant, and measuring the change in O.D.(460nm) for one minute. (Settings on recorder were range = 1000, speed = 1cm/min).

The change in O.D. per minute was multiplied by 2.65 to determine the peroxidase units.

g. Indicator substrate was 100ml phosphate buffer (0.6M, pH 6.0), 0.017ml of 30% H_2O_2, and 1.67mg/ml dianisidine (0.0167 g/100ml buffer).

h. Protein content was measured using the commercially available Lowry's test kit (Sigma Chemical Co., St. Louis, Missouri).

Table I sets forth the relative hemolytic and cytotoxic activity of fractions expressed in percent hemolysis or cytotoxicity. The term O.D. represents the optical density of the fraction at a wavelength of 280nm. The measurement is linearly related to the protein concentration of the fraction.

Table I

Fraction Tube	% Hemolysis	% Cytotoxicity	O/D.
1	3.5	5.8	0.03
2	3.5	4.31	0.01
3	3.5	0	0.02
4	3.5	14.89	0.002
5	3.5	7.42	0.00001
6	3.5	0	0.04
7	3.5	0	0.06
8	3.5	0	0.03
9	3.5	5.69	0.1
10	3.5	5.23	0.18
11	3.5	1.36	0.2
12	7.8	9.86	0.19
13	15.7	0.81	0.2
14	15.7	0	0.2
15	15.7	5.3	0.183
16	15.7	2.9	0.152
17	11.4	5.7	0.15
18	8.28	5.4	0.12
19	8.28	8.86	0.11
20	8.28	59.1	0.08
21	8.28	22.7	0.1

Sensitivity to Zinc

Solutions of 2mM of lead acetate, ferric chloride, zinc sulfate, stannous chloride or aluminum hydroxide in McCoy's media were added to sterile filtrates and incubated for 30 minutes before testing for their effect on the cytotoxic and hemolytic activities.

M. bovis culture filtrates were then tested for cytotoxic activity against bovine peripheral blood neutrophils and for hemolytic activity by the tests described above.

Zinc sulfate completely inhibited the cytotoxic activity of the filtrates, but had no effect upon the hemolytic activity. None of the other solutions affected the cytotoxicity of the filtrates.

Casein Hydrolysis Study

This test was done to determine whether the cytotoxin could hydrolyze protein, casein in particular. Protease activity of the cytotoxin was measured in Trypticase Soy Agar plates containing 0.5% autoclaved skim milk. Ten microliters of the cytotoxin were added to 3mm wells within the agar. The plates were examined for zones of milk proteolysis after 24 hours. No clear zones occurred around the cytotoxin wells indicating that the cytotoxin was unable to hydrolyze casein.

D. Vaccine Preparation and Administration

1. Precipitate crude M. bovis filtrate with 70% ammonium sulfate for 4 hours.
2. Centrifuge pellet at 20,000xg for 1 hour.
3. Resuspend pellet in Hepes buffer (0.02M, pH 7.4).
4. Pass through Bio-Gel PD-10 column, collect first protein peak and dilute 1:10 in Hepes buffer.
5. Absorb to DEAE Sephacryl® column.
6. Elute with 0.3M NaCl in Hepes buffer.

7. Mix eluate 1:1 with adjuvant.

8. Inject 1ml of the mixture subcutaneously into an adult cow.

WHAT IS CLAIMED IS:

1. A cytotoxin from Moraxella bovis substantially free of intact microbial cells, and possessing the following characteristics:

(a) cytotoxic to bovine peripheral blood neutrophils; and

(b) lacks hemolytic activity.

2. The cytotoxin of claim 1, wherein the cytotoxin is not capable of hydrolyzing casein.

3. The cytotoxin of claim 1, wherein the cytotoxic activity to bovine peripheral blood neutrophils is sensitive to zinc salts.

4. The cytotoxin of claim 1, wherein the cytotoxin is net negatively charged at a pH of 7.4.

5. The cytotoxin of claim 1, further characterized by its ability to elute off of a DEAE exchange column with a 3ml gel bed under a salt gradient at a 0.2M to about a 0.3M salt concentration.

6. A vaccine for protection of animals from Moraxella bovis comprising a cytotoxin from M. bovis substantially free of intact microbial cells, which is cytotoxic to bovine peripheral blood neutrophils and which lacks hemolytic activity.

7. The vaccine of claim 6, wherein the cytotoxin is not capable of hydrolyzing casein.

8. The vaccine of claim 6, wherein the cytotoxin is further characterized by its ability to elute off of a DEAE column under a salt gradient at a 0.2M to about a 0.3M salt concentration.

9. A method for protecting animals from infection of M. bovis which comprises the vaccination of the animals with a vaccine comprising a cytotoxin substantially free of intact microbial cells derived from M. bovis which is cytotoxic to bovine peripheral blood neutrophils and which lacks hemolytic activity.

10. The method of claim 9, wherein the cytotoxin is further characterized by its ability to elute off of an anion exchange column under a salt gradient at a 0.2M to about a 0.3M salt concentration.

11. A vaccine for protection of animals from infectious bovine keratoconjunctivitis comprising a cytotoxin substantially free of intact microbial cells the cytotoxin from M. bovis and characterized by:

(a) its ability to elute off of a DEAE exchange column having a 3ml gel bed at about a 0.2M to about 0.3M salt concentration; and

(b) upon elution from the column is cytotoxic to bovine peripheral blood neutrophils and lacks hemolytic activity.

12. The vaccine of claim 11, wherein the cytotoxin is not capable of hydrolyzing casein.

13. The vaccine of claim 11, wherein the cytotoxin is net negatively charged at a pH of 7.4.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00106

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 15/00, 15/02, 15/04; A61K 39/02, 37/48
U.S. CL.: 530/350; 424/88,92

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. CL.	530/350 424/88,92

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Data bases: Chemical Abstract Services ONline (1967-1990), File CA; File Biosis). Automated Patent Searching (1975-1990). Search Terms: Moraxella bovis, Toxin Cytotxin

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X T Y	American Journal of Veterinary Research, Volume 51(2), issued February 1990, Hoien-Dalen et al.,"Comparative Characterization of the Leukocidic and Hemolytic Activity of Moraxella bocis ," pages 191-196. See entire document.	1-13 1-13
X P Y	American Journal of Veterinary Research, Volume 50(1), issued January 1989, Kagonyera et al. "Effects of Moraxella bovis and Culture filtrates on ^{51}Cr-labeled Bovine Neutrophils,"pages 18-21. See the Disussion pages 20-21.	1-5 1-13
X Y	Conference of Research Works in Animal Discase, Abstract Number 49, Published 1988, Hoicn-Dalen et al., "Partial Characterization of a Moraxella bovis Leukocidin and Comparison to the M. bovis Hemolysin, "page 9. See the entire abstract.	1-5 1-13
Y	Canadian Journal of Comparative Medicine, Volume 37, issued January 1973, Pugh et al., "The Pathophysiological Effects of Moraxella bovis Toxins on Cattle, Mice, an Guinea Pigs, "pages 70-78. See the Abstract and pages 76-77.	1-13

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 April 1990	03 MAY 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	R. Keith Baker

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	American Journal of Veterinary Research, Volume 36(3), Issued March 1975, Hughes et al., "Experimentally Induced Infectious Bovine Keratoconjunctivitis; Relationship of Vaccination Schedule to Protection Against Exposure with Homologous Moraella bouis Culture," pages 263-265. See the Summary and the last paragraph.	6-13
Y	WO,A 86/06635 (Biotechnology Australia Pty, LTD.) 20 November 1986 (20.11.86) See the Abstract claims 12 and 13.	6-13

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. x] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Group I, Claims 1-5, drqwn to a cytotoxin from Moraxella bovis
Group II, Claims 6-13, drawn to a caccine for protection of animals from Moraella bovis and a method of using the vaccine

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to Form PCT/ISA/210, Part VI.

Reason for holding lack of unity of invention:

The invention as defined by Group II (claims 6-13), classified in class 424, subclass 92, is drqwn to a vaccine. Group I (claims 1-5) is drawn to a cytotoxin, classified in class 530 subclass 350. A vaccine (Group II) and a cytotoxin (Group I are distinct products having a separate classification and recognized divergent subject matter. Additionally, the cytotoxin can have other uses other than its use in a vaccine. Jurthermore, PCT Rule 13.2 allows applicants to claim a (one) product; however, the two groups listed above represent two products.